ERDC/TN APCRP-BC-13
September 2009

Storage Stability of Dried Microsclerotia of the Biological Control Pathogen *Mycoleptodiscus Terrestris*

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PURPOSE: This technical note describes storage stability of the biological control pathogen *Mycoleptodiscus terrestris* (Gerd.) Ostazeski (Mt). Dried microsclerotia from four different fermentation batches were harvested and stored at 4º C. After varying amounts of time in storage, they were tested for efficacy against the invasive macrophyte hydrilla (*Hydrilla verticillata* (L.f.) Royle).

INTRODUCTION: The biological control pathogen, Mt, is currently being researched as a potential bioherbicide for management of the submersed macrophyte hydrilla. The pathogen was isolated from hydrilla plant tissue collected in a canal adjacent to Sheldon Reservoir, TX in the early 1990's. Subsequent testing found that when applied as liquid slurry, the isolate was efficacious alone or in combination with chemical herbicides for hydrilla management (Shearer 1997, 1998; Netherland and Shearer 1996; Nelson and Shearer 2005; Nelson et al. 1998; Shearer and Nelson 1999, 2002).

In developing Mt as a bioherbicide, emphasis has been placed on the production of stable propagules (microsclerotia) that are highly resistant to desiccation (Cooke 1983; Coley-Smith and Cooke 1971) rather than thin-walled spores (conidia) or hyphal units (Shearer and Jackson 2006). This approach was taken in large part because the fungal isolate readily produces melanized hyphal aggregates (i.e., microsclerotia) on standard laboratory media such as potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) but does not conidiate (Shearer and Jackson 2003, 2006). In a liquid broth culture medium, microsclerotia can be induced to develop over a four-day fermentation period (Shearer and Jackson 2006). The microsclerotial propagules are then harvested through a dewatering process, air dried to a moisture content between 5 and 10 percent, vacuum packed, and stored at 4 °C.

In nature, microsclerotia serve as survival propagules as well as reproductive organisms in a great many fungal species (Webster and Weber 2007). They have been reported as capable of surviving for long periods (Coley-Smith and Cooke 1971), and when conditions are favorable they can germinate by the development of mycelium (myceliogenic germination), by asexual spores (sporogenic germination), or by sexual fruit bodies (carpogenic germination) (Webster and Weber 2007). Plating of dried microsclerotia of Mt onto water agar revealed they were capable of germinating myceliogenically (germ tube formation) within 24 hr and quite unexpectedly sporogenically (production of conidial masses on the surface of the microsclerotium) 48 hr later (Shearer and Jackson 2006). Once sporogenic germination began, it continued over a 5- to 6-day period with a new crop of spores produced daily (Shearer and Jackson 2006).

Dried microsclerotia inoculum also proved to be efficacious on hydrilla (Shearer 2009). Sprinkled over the water surface of 55-L aquaria containing rooted hydrilla plants that had formed a canopy,

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
SEP 2009		**00-00-2009 to 00-00-2009**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Storage Stability of Dried Microsclerotia of the Biological Control Pathogen Mycoleptodiscus Terrestris	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Engineer Research and Development Center,Environmental Laboratory,3909 Halls Ferry Road,Vicksburg,MS,39180-6199**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**6**	

the microsclerotia gradually rehydrated and began to fall through the water column. As the particles fell, they impinged on leaf surfaces and in leaf axils of hydrilla. Approximately seven days post-inoculation, disease onset (i.e., chlorosis) was visibly apparent. As disease progressed, stem and leaf tissues became flaccid and hydrilla shoot biomass collapsed to the bottom of the aquariums by 14 days post-inoculation.

The capability of microsclerotia to germinate both myceliogenically and sporogenically upon rehydration enhances the potential of Mt for use as a non-chemical biological control agent for hydrilla. While drying the fungus does not appear to impact efficacy, it is unknown how prolonged storage might affect the viability and virulence of the organism. Because shelf life is a primary consideration of bioherbicide marketability, studies were undertaken to test efficacy of dried material after various periods of cold storage.

MATERIALS AND METHODS

Fungal Inoculum. Stock cultures of Mt (NRRL #30559) were stored and plated as described in Shearer and Jackson (2006). All fermentation and dry inoculum preparation were carried out at the United States Department of Agriculture National Center for Agricultural Utilization Research (USDA-NCAUR, Peoria, IL). Fermentation batches of Mt microsclerotia were harvested, air-dried overnight, vacuum packed in polyethylene bags, and stored at 4 ºC on 3/6/07, 5/15/07, 6/13/07, and 7/11/07. Dry material was shipped overnight in refrigerated containers to the U.S. Army Engineer Research and Development Center (ERDC), Vicksburg, MS for bioassays. As needed, the dried material was weighed out into plastic weigh boats for aquarium efficacy testing on hydrilla (see below) and for spore production bioassays. After use, the polyethylene bags were resealed with a vacuum packaging machine (Henkelman H100, Hertogenbosch, Netherlands) and stored at 4 ºC.

Microsclerotia Bioassays. For microsclerotia yield data, 100 µl of a 10^{-1} dilution of the fermentation broth was placed on a glass slide, overlaid with a coverslip, and all well-formed microsclerotia on the slide were counted. During sampling, microsclerotium suspensions were constantly vortexed to ensure homogeneity.

Microsclerotia viability following drying was determined by sprinkling a minimum of 100 dried microsclerotia onto the surface of water agar plates (20 g agar; 1 L H_2O). Following incubation for 24 hr in the dark at 28 ºC, 100 microsclerotia were examined microscopically for myceliogenic germination as a measure of viability.

To assess spore production, three 25-mg aliquots of dry Mt were sprinkled onto water agar plates and incubated in the dark at 28 ºC for 7 days. The plates were flooded with sterile water and the spores dislodged from the microsclerotia with a sterile loop. The water containing the spores was measured to determine final volume and the spores were counted using a hemacytometer. Total number of microsclerotia spores per gram was determined by multiplying the spore count by the final volume of liquid and dividing the product by the weight of the sample (0.025 g).

Aquarium Bioassays. Studies were conducted in 55-L aquariums located in a controlled-environment growth chamber at the ERDC. Conditions in the growth chambers were maintained for optimal hydrilla growth: 25 $\pm$ 1 C and a 14:10-hr light-dark photoperiod. The aquariums (0.9 m tall $\times$ 0.09 m^2) were filled with a water-based culture solution (Smart and Barko 1984). Lake sediment

collected from Brown's Lake at the ERDC, was amended with ammonium chloride (0.5 g L^{-1}) and Esmigran (1.7 g L^{-1}) (Scotts, Marysville, OH). Four plastic cups (0.95 L) filled three-fourths with amended lake sediment were planted with five 15-cm apical cuttings from dioecious hydrilla, overlain with silica sand to prevent sediment and nutrient dispersion into the water, and placed in each aquarium. The hydrilla was obtained from culture ponds at the U.S. Army Engineer Lewisville Aquatic Ecosystem Research Facility (LAERF), Lewisville, TX. Air was gently bubbled in each aquarium to provide circulation. The plants were allowed to grow in the aquariums for approximately 28 days, by which time they formed a canopy.

Following each fermentation run, an aquarium bioassay was conducted. The dry inoculum was applied by sprinkling it evenly onto the water surface and allowing it to naturally dissipate over the hydrilla. As the rehydrated granules fell through the water column they became lodged on leaves and in leaf axils. All initial treatments included Mt applied at an effective rate of 0.04 g L^{-1} following dilution and an untreated control. Each treatment was replicated four times. At 28 days after treatment (DAT), hydrilla shoot biomass was harvested, dried for 4 days at 60 ºC to a constant weight, and dry weight was recorded.

Dried material from the four different fermentation batches was retrieved from storage on 9/12/07 and again on 11/15/07 for retesting on rooted hydrilla in the 55-L aquaria. In these two subsequent bioassays, treatments included effective rates of 0.02 g L^{-1} and 0.04 g L^{-1}dry Mt following dilution, and an untreated control. Each treatment was replicated three times. Dry Mt application and hydrilla harvesting were conducted as described above.

Statistics. Analysis of variance (ANOVA) (Statistica 8.0, Stat Soft, Tulsa, OK) was used for statistical treatment of data. Mean separations were accomplished using Tukey's Honest Significant Difference (HSD) test. Test of significance was conducted at $p \leq 0.05$.

RESULTS AND DISCUSSION: Microsclerotia numbers in the fermentation broth were enumerated on day 4 just prior to liquid culture harvest at NCAUR. Although the same isolate, media, and procedures were used for all the batches, significantly higher numbers of microsclerotia formed in the June batch than in batches produced in March, May, or July ($p = 0.0097$) (Table 1). Examination of microsclerotia numbers from other batches revealed that numbers do vary from batch to batch, and the numbers found in the current studies are well within the range of those recorded over the past two years (unpublished data).

Analysis of microsclerotia for percent myceliogenic germination was undertaken immediately after drying of each batch but prior to placement in cold storage. Microsclerotia from the batch produced in May germinated with higher frequencies after 24 hr incubation than microsclerotia produced in May, June, or July ($p = 0.00023$) (Table 1). Myceliogenic germination did not prove to be an indicator of sporogenic germination of dried material. For example, the batch with the lowest myceliogenic germination (i.e. the June batch) produced an equivalent number of spores as the batch with the highest myceliogenic germination rate (i.e., the May batch). It should be noted that myceliogenic germination percentages were determined 24 hr after plating of the dry granules, and the plates were not checked again for any delayed germination of the microsclerotia. Unfortunately, it is not possible to accurately read the plates if they are incubated more than one day because the colonies that develop from each microsclerotium tend to overgrow one another.

Table 1. Microsclerotia counts/ml of fermentation broth, myceliogenic germination, and sporogenic germination per gram dried *Mycoleptodiscus terrestris* from fermentation runs in March, May, June, and July. Means followed by a different letter are significantly different according to Tukey's Honest Significant Difference Test (TDS) at p ≤ 0.05.

Month of production	Microsclerotia #/ml[1]	Percent Microsclerotia Germination[1]	Spore count spores/g[1]	Percent Microsclerotia Germination[2]	Spore count spores/g[2]	Percent Microsclerotia Germination[3]	Spore count spores/g[3]
March	1.9×10^3a	91.5b	2.3×10^6a	88.0ab	4.10×10^7a	97.5a	4.55×10^7a
May	3.4×10^3a	100.0a	9.7×10^7b	96.5a	1.05×10^8a	96.0ab	9.85×10^7a
June	1.0×10^4b	84.0c	9.1×10^7b	79.0b	9.05×10^7a	92.5b	9.40×10^7a
July	4.8×10^3a	89.5b	4.0×10^6a	98.0a	1.03×10^7a	92.5b	3.60×10^7a

[1] Initial analysis of microsclerotia from fermentation batches produced in March, May, June, and July.
[2] Myceliogenic and sporogenic germination rates of microsclerotia plated in September 2007.
[3] Myceliogenic and sporogenic germination rates of microsclerotia plated in November 2007.

Time in storage prior to initial efficacy testing on rooted hydrilla varied from one day to approximately one month (Table 2). For consistency, it would have been desirable to test each batch immediately after production. However, it was not always possible to have plants at the proper growth stage for testing. Preliminary testing of dried Mt on plant material showed the March batch to be significantly less efficacious on hydrilla than batches produced in May, June, and July ($p = 0.0045$) (Table 2).

Table 2. Percent hydrilla shoot biomass reduction in 55-L aquariums when treated with dry *Mycoleptodiscus terrestris* microsclerotia. Means followed by a different letter are significantly different according to Tukey's Honest Significant Difference Test (TDS) at p ≤ 0.05.

Month of production	Time in storage prior to first use	Percent mean biomass reduction, 0.04g L^{-1*}	Months in storage	Percent mean biomass reduction		Months in storage	Percent mean biomass reduction	
				0.02g L^{-1*}	0.04g L^{-1*}		0.02g L^{-1*}	0.04g L^{-1*}
March	1 day	75.3a	7	99.4a	98.4a	9	94.3a	98.6a
May	7 days	91.4b	5	95.7a	98.3a	7	81.7a	95.3a
June	14 days	89.9b	3	95.7a	98.5a	5	56.5b	90.3a
July	30 days	100.0b	2	97.8a	99.9a	4	82.1a	98.9a

[*] Effective application rate following dilution in water.

When simultaneous testing of the four batches was undertaken in September 2007, dry Mt granules had been in storage for approximately 7, 5, 3, and 2 months, respectively, for batches produced in March, May, June, and July. The same 0.04 g L^{-1} effective inoculation rate was used in the aquarium study, so efficacy comparisons could be made between batches and time in storage. An additional effective rate of 0.02 g L^{-1} was added to the treatment regime because the July batch resulted in 100 percent reduction in hydrilla shoot biomass at the 0.04 g L^{-1} inoculation rate in the initial bioassays (Table 2). All batches and treatment rates used during the September testing worked extremely well and reduced hydrilla shoot biomass by more than 90 percent. There were no significant differences in hydrilla shoot biomass reductions between batches or treatment rates (Table 2). Myceliogenic germination of microsclerotia on water agar after various periods in storage was again not a good indicator of Mt spore production or efficacy. Although there were significant differences in myceliogenic germination between batches during the September bioassay, there were

no significant differences in sporogenic germination of the microsclerotia (Table 1) or efficacy on hydrilla (Table 2).

At the time of the final bioassay, dry Mt granules had been in storage for approximately 9, 7, 5, and 4 months, respectively, for batches produced in March, May, June, and July. Applied at the effective rate of 0.02 g L^{-1}, Mt was less efficacious on hydrilla than in the September bioassay (Table 2). The batch produced in June had significantly lower efficacy on hydrilla than any of the other batches ($p = 0.00187$). In terms of management, a treatment strategy that results in less than 80 percent control of plant biomass can be considered an unsuccessful operational treatment. Even when percent control is slightly greater than 80 percent, there is a high probability that hydrilla could successfully regrow from undamaged stems and/or root crowns. Applied at the higher rate in the final bioassay, there were no significant differences between batches in terms of efficacy on hydrilla. Further, there were no significant differences in efficacy at the higher rate when comparing hydrilla shoot biomass reductions between the September and November bioassays.

At the time of the final bioassay, the batches that had been in storage the longest had higher myceliogenic germination than more recent batches (Table 1). As for sporogenic germination, there were no significant differences among batches at the time of the final bioassay and overall little change in spore production per gram dried Mt from the September bioassay.

Few studies have examined the effects of cold storage on fungal microsclerotia. It has been documented that sclerotia of *Claviceps purpurea* require a chilling period before enzymes capable of mobilizing the lipid reserves contained within the sclerotia can develop (Webster and Weber 2007). Budge et al. (1998) determined that *Sclerotinia sclerotiorum* sclerotia did not necessarily require low soil temperature (< 10 ºC) to break constitutive dormancy but cold treatment did stimulate carpogenic germination. In the present study, it is unknown if cold treatment stimulated any enzymatic activity but a period of cold storage, even as little as 7 days, seemed to increase efficacy of the Mt inoculum on hydrilla (Table 2). This was somewhat surprising in that cold storage did not seem to dramatically improve the ability of the microsclerotia to germinate myceliogenically nor sporogenically on water agar (Table 1). Warm water temperatures (25 ºC ± 1 ºC) and the presence of a host plant may have affected both germination and sporulation of the microsclerotial inoculum. Microscopic examination of some of the microsclerotia that remained floating on the water surface of the aquariums revealed that copious amounts of spores were present on the surface of the microsclerotia (personal observation). Release of these spores into the water would in all likelihood provide secondary infective propagules.

The present research has demonstrated that dried Mt can remain stable in cold storage for at least nine months and efficacy may even improve after one month in cold storage (Table 2). This has important implications in strategies for developing and using Mt as a bioherbicide. The product could be produced and placed in cold storage starting in November and would be available for use as early as February, when hydrilla starts to regrow in parts of Florida. A shelf life of nine months would be more than sufficient to allow the delivery and use of the bioherbicide prior to water temperatures exceeding 30 ºC after which fungal growth and infectivity of Mt becomes severely limiting.

FUTURE WORK: Cooperative efforts between ERDC and USDA-NCAUR are aimed at continuing to improve stability and efficacy of a dried mycoherbicide product that can be used for hydrilla management.

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> Shearer, J. F. 2009. *Storage stability of dried microsclerotia of the biological control pathogen Mycoleptodiscus terrestris*. APCRP Technical Notes Collection. ERDC/TN APCRP-BC-13. Vicksburg, MS: U.S. Army Engineer Research and Development Center.

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